Population genetic diversity and regional differentiation of Chinese forest frogs (*Rana chensinensis*) in Heilongjiang Province

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Abstract: The Random Amplified Polymorphism DNA (RAPD) markers were used to study the intra-specific diversity and regional differentiation of the Chinese forest frogs (*Rana chensinensis*), which were sampled from the fields of 8 regions in Heilongjiang Province. Totally 78 polymorphic DNA loci were amplified by 10 RAPD primers. By genetic distance analysis and phylogenetic tree reconstruction with the Neighbor-Joining (NJ) method the results showed that the populations of Chinese forest frogs distributed in 8 regions existed great differentiation (Average Fst=0.347, SD=0.235) while there was the paradox between geographic distances and genetic distances. Based on geographic and geological data, a hypothesis was posed that it is very possible that the hilly lands in the downstream of the Songhua River and the Heilong River were the center of the origin of the Chinese forest frogs in Heilongjiang Province. And mainly through the Songhua River system, the Chinese forest frogs dispersed into the Songneng Plain from the Sanjiang Plain whereas the Fangzheng region became a sub-center for the western dispersion.

Key words: Chinese forest frogs (*Rana chensinensis*); Population genetics; Regional differentiation
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Introduction

The Chinese Forest frog (*Rana chensinensis*), so-called Hashima, is one of rare traditional medical animals in the Southeast Asia. In China, this species disperses in the northeastern areas, the northwestern and the northern areas. And recent studies were mainly centered on following aspects such as biological characteristics (Li *et al.* 1998), comparative karyotype study (Wu 1981, 1982; Luo 1985), histological and histo-chemical study of the oviduct (He *et al.* 1986), isoenzyme and its codon gene (Zhang *et al.* 1996), fecundity and geographic variation (Lu 1994), and the artificial reproduction technique (Liu 1989), etc..

Based on background of high sensitivity of the Polymerase Chain Reaction (PCR), and its first appearance at the beginning of 1990's, the Random Amplified Polymorphism DNA (RAPD) technique provided a new marker for genetic researches on those organisms with few genetic data and little information (Williams *et al.* 1990). In this paper, RAPD markers were used in the study of intra-specific diversity and regional differentiation of the Chinese forest frogs that collected from the fields of eight regions in Heilongjiang Province. And a hypothesis about the phylogenetics of the Chinese forest frogs in Heilongjiang Province was posed.

Materials and methods

Materials and DNA extraction

Muscular tissues of 39 individuals of the Chinese forest frogs were obtained from the fields of eight regions in Heilongjiang Province. Their serial numbers and collecting sites were showed in Table 1.

About 1g of muscular tissue was cut from each frog and placed in a 1.5-mL tube. 500 μL lysis solution (10 mmol/L Tris-HCl, 10 mmol/L EDTA, pH 8.0, 1.0% SDS, 200 μg/mL Proteinase K) was added.

Then tissues were incubated in 37 °C overnight until tissues lysed completely. Extracted DNA by phenol/chloroform method and precipitated DNA with absolute ethanol followed washing of 70% ethanol. Finally, DNA pellets were solved by 200 μL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and stored in 4 °C.

RAPD amplification and electrophoresis

RAPDs primers were synthesized by the Laboratory of Animal Genetics and Breeding of the College of Wildlife Resources of the Northeast Forestry University.

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Table 1. Sampling sites and sample number of Chinese forest frogs

Sampling sites	Sample NO. of individual
Shanhe	1 2 3 4 5
Chaihe	6 7 8 9 10
Wudihe	11 12 13 14 15
Dongfanghong	16 17 18 19 20
Yichun	21 22 23 24
Shangzhi	25 26 27 28 29 30
Suiyang	31 32 33 34 35
Fangzheng	36 37 38 39

The sequences of these primers were showed in Table 2. Amplification was performed in 10 mmol/L Tris-HCl, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 100μmol/L dNTPs, 15ng primer, 1U *Taq* polymerase with 30 ng of template DNA per 25 μL reaction. After an initial denaturation of 1 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C were performed, followed by the final 7 min extension at 72 °C (Williams *et al.* 1990). The PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus). PCR products were analyzed by electrophoresis in 2% agarose gel (Promega Inc.) in 0.5x TBE buffer (90 mmol/L Tris borate, pH8.3, 2 mmol/L EDTA). Electrophoresis was carried out at room temperature at 2 v/cm for 4-5 h. Gel was stained by E.B. and photographed on UV light (Fig. 1).

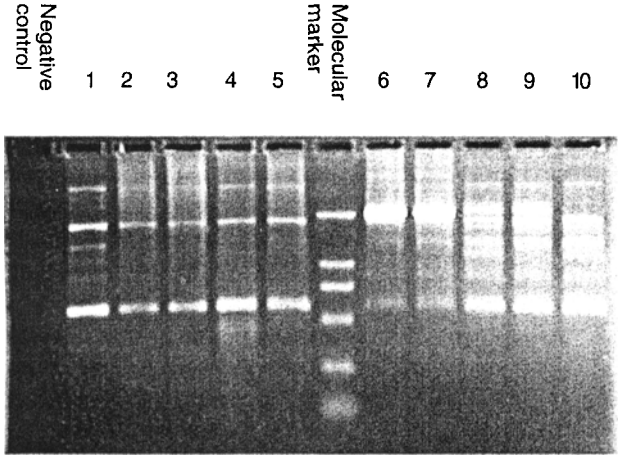

Fig. 1 Electrophoresis of RAPD products by primer B-14

Statistical analysis

DNA bands were scored as binary data (0 or 1), and the Hillis correction of Nei's Distance (Swofford *et al.* 1996) was calculated. After that, the Neighbour-joining tree was reconstructed by MEGA 2.0b (Sudhir *et al.* 2000). Meanwhile the binary data also were used to *F*-statistics to measure the degree of differentiation among the eight populations by RAPDs 1.0 (Apostol 1993).

Results

In our experiment, 10 primers were selected for clear and reproductive bands, and total 78 dominant loci were amplified. Although only 5-6 individuals were obtained from each sampling site, we had detected 78 loci and the statistical results of these data were reliable and unbiased according to researcher of Nei (1978).

Table 2. Ten RAPD primers and their sequences

Primer code	Sequences of primers 5'→3'	Primer code	Sequences of primers 5'→3'
A-01	CAGGCCCTGG	F-06	GGGAATTCGG
A-04	AATCGGGCTG	G-16	AGCGTCCTCC
B-13	TTCCCCCGCT	H-05	AGTCCTTCCC
B-14	TCCGCTCTGG	H-09	TGTAGCTGGG
B-15	GGAGGGTGTT	I-17	GGTGGTGATA

The average genetic distance among eight populations was 0.215 (SD=0.042); and the Neighbor-joining tree on the genetic distance matrix was reconstructed and showed in Fig. 2. Meanwhile, the average Fst of the eight populations was 0.347 (SD=0.235) the result showed that there had great differentiation among different frost frog populations from eight regions of Heilongjiang Province. During data analysis, other indexes were also used and the UPGMA tree was reconstructed too (unpublished), they all showed similar results.

According to Fig. 2, the most interested feature was that the genetic distances among the eight populations did not tally with their geographic distances. For example, there existed the closest geographic distance but the farthest genetic distances existed between the populations in Chaihe and Suiyang; whereas the populations in Shanhe, Suiyang and Wudihe had the closer genetic distances compared with the farther geographic distances. Furthermore, skipping the population in Fangzheng, the populations in Liangshui and Shangzhi owned closer genetic distance, and the population in Fangzheng was clustered into the subgroup of the populations in Chaihe and Dongfanghong. In fact, the Laoye Mountain separated Fangzheng into Dongfanghong and Chaihe.

Discussion

For a long time, it had been noticed that the Chinese frost frogs from different regions of Heilongjiang Province had different pharmaceutical function even they were one species. Referenced from the geographic and geological data, a hypothesis was posed as follows:

1) In the range of Heilongjiang Province, the hilly area in the downstream of the Songhua River and the Heilong River surrounded by the Zhangguangcai Mountains, the Laoye Mountain and the Wusuli River were very probably the center of the origin of the Chinese forest frogs in Heilongjiang Province. From this central area, the Chinese forest frogs began to disperse into surrounding areas. And the population of the Chinese forest frogs in this area (Dongfanghong and Chaihe) should show more complex diversity. This point also was supported by the fact that only the individuals from these two sites (Dongfanghong and Chaihe) could not be well clustered together when frog individuals were regarded as OTUs respectively (data unpublished).

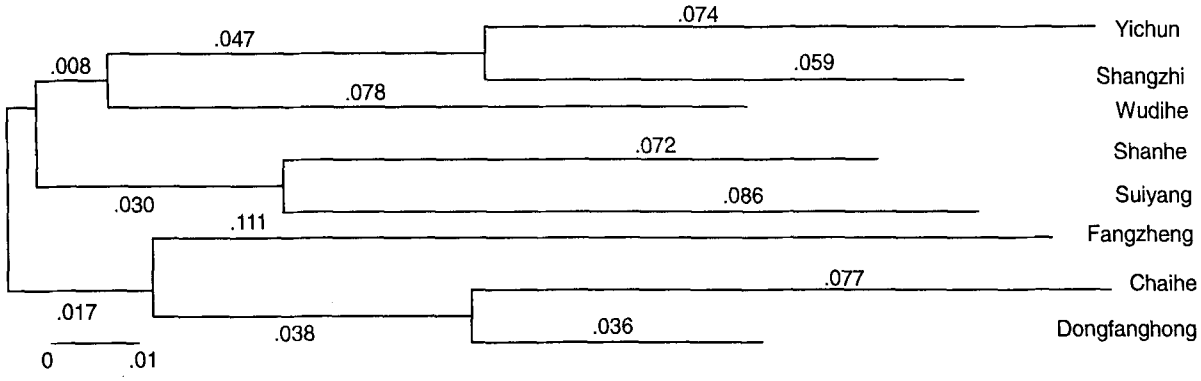

Fig. 2 Phylogenetic tree of the Chinese forest frogs from eight regions of Heilongjiang Province based on genetic distances

Notes: This is an unrooted phylogenetic tree, the number at the end of each branch represents one population from coresponsible sampling sites , and the right column to the tree is the name of the sampling sites

2) When migrating into the Songneng Plain, it was possible through the way of the Songhua River system along Jiamusi, Yilan and Fangzheng that the Chinese forest frogs skirted around the Zhangguangcai Mountains and the Xiaoxing'an Mountains. Consequently, the region of Fangzheng became a sub-center of the western dispersion. That was why the population of Fangzheng was clustered closer with the populations of Chaihe and Dongfanghong.

3) In the following dispersion on the Songneng Plain, the migration was affected by the Human beings' action and became slowly.

4) By the Heilongjiang River system, the Chinese forest frogs migrated into Wudihe area of the Xunke region from the center of the origin.

5) It was the Laoye Mountain that makes the populations of Chaihe and Suiyang owned the farthest genetic distances, which compared with the closest geographic distances.

It should be pointed out that it was the joint function of the dispersion and the random drift in the population that formed present population structure of the Chinese forest frogs in Heilongjiang Province, and all deduction above was based on preliminary study and needed further evidences.

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